PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

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PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2004/031912	29.09.2004	03.10.2003

International Patent Classification (IPC) or both national classification and IPC
C12N15/10, C12N15/64, C12N15/66, C12N15/70, C12N9/22, G06F19/00

Applicant
PROMEGA CORPORATION

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

02/28/06
D- RESP. DUE

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2004/031912

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☒ contained in the international application as filed.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date (Rules 43*bis*.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees, the applicant has:

   ☒ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ not paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is

   ☐ complied with

   ☒ not complied with for the following reasons:

   see separate sheet

4. Consequently, this report has been established in respect of the following parts of the international application:

   ☒ all parts.

   ☐ the parts relating to claims Nos.

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6-13,19-25,27-38,40-43,47,48,52-58,62,63,66-70,72,74-77,82-85,88-100,104,107,131-140
	No:	Claims	1-5,14-18,26,39,44-46,49,50,51,59-61,64,65,71,73,78-81,86,87,101-103,105,106,108-130,141
Inventive step (IS)	Yes:	Claims	6-13,19-25,27-36,40,47,48,52-58,62,63,66-70,72,74-77,82-85,98,104,107,131-140
	No:	Claims	1-5,14-18,26,37-39,41-46,49,50,51,59-61,64,65,71,73,78-81,86-97,99-103,105,106,108-130,141
Industrial applicability (IA)	Yes:	Claims	1-141
	No:	Claims	

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item IV.

The separate inventions/groups of inventions are:

Claims: 1-26, 28-35, 36-partially, 40

A **method for the directional subcloning of DNA fragments** comprising: a) providing a first vector comprising a first selectable marker gene and a DNA sequence of interest, which DNA sequence of interest is flanked by at least two restriction enzyme sites (Res), wherein at least one of the flanking RE sites is a site for a first RE which has infrequent restriction sites in cDNAs or open reading frames from at least one species and generates complementary single-strand DNA overhangs, wherein at least one of the flanking RE sites is for a second RE which has infrequent restriction sites in cDNAs or ORFs from at least one species and generates ends that are not complementary to the overhangs generated by the first RE, wherein digestion of the first vector with the first RE and the second RE site generates a first linear DNA fragment which lacks the first selectable marker gene but comprises the DNA sequence of interest; b) providing a second vector comprising a second selectable marker gene which is distinguishable from the first selectable marker gene and non-essential DNA sequences, which non-essential sequences are flanked by at least two restriction enzymes sites, wherein at least one of the flanking RE sites in the second vector is for a third RE which generates complementary single-strand DNA overhangs that are complementary to the single-strand DNA overhang generated by the first restriction enzyme in the first linear DNA fragment, wherein at least one of the flanking RE sites in the second vector is for a fourth RE which generates ends that are not complementary to the ends generated by the first or third RE but can be ligated to the ends generated by the second RE, and wherein digestion of the second vector with the third RE and the fourth RE generates a second linear DNA fragment which lacks non-essential DNA sequences but comprises the second selectable marker, which second linear DNA fragment is flanked by ends which permit the oriented joining of the first linear DNA fragment to the second linear DNA fragment; and c) combining the first and second vectors, the first vector and the second linear DNA fragment, or the second vector and the first linear DNA fragment in a suitable buffer with one or more REs under conditions effective to result in digestion to yield a mixture comprising the first and second linear DNA molecules which are joined in an oriented manner. said method wherein at least one

hapaxoterministic RE is used;

Claims 27

A method for producing a vector suitable for expression of an amino acid sequence of interest, comprising: combining at least two vectors in a suitable buffer with one or more restriction enzymes and optionally DNA ligase under conditions effective to result in digestion and optionally ligation to yield a mixture optionally comprising a third vector, wherein a first vector comprises a first selectable marker gene and a DNA sequence of interest, which DNA sequence of interest is flanked by at least two restriction enzyme sites, wherein two or more of the flanking restriction enzyme sites are sites for a **first restriction enzyme which is a hapaxoterministic restriction enzyme**, wherein digestion of the first vector with the first restriction enzyme generates a first linear DNA fragment which lacks the first selectable marker gene but comprises the DNA sequence of interest and a first pair non-self complementary single-strand DNA overhangs, wherein a second vector comprises a second selectable marker gene which is distinguishable from the first selectable marker gene and non-essential DNA sequences that optionally include a counterselectable gene, which non-essential DNA sequences are flanked by two or more restriction enzyme sites, wherein two or more of the flanking sites in the second vector are for a **second restriction enzyme which is a hapaxoterministic restriction enzyme**, wherein digestion of the second vector with the second restriction enzyme generates a second linear DNA fragment which lacks non-essential DNA sequences but comprises the second selectable marker gene and a second pair of non-self complementary single-strand DNA overhangs, wherein each of the second pair of the non-self-complementary DNA overhangs is complementary to only one of the single-strand DNA overhangs of the first pair of non-self complementary single-strand DNA overhangs and permits the oriented joining of the first linear DNA fragment to the second linear DNA fragment.

Claims: 37-38

A method of inducing expression of a DNA sequence of interest in a host cell, comprising contacting a recombinant host cell which is **deficient in rhamnose catabolism**, and has a recombinant DNA molecule comprising a rhamnose-inducible promoter operably linked to an open reading frame for a heterologous RNA polymerase, with rhamnose and an expression vector comprising a promoter for the heterologous RNA polymerase operably linked to a DNA sequence of interest.

Claim: 39

A method comprising introducing a vector comprising a **nucleic acid fragment encoding a barnase** which lacks a secretory domain into a recombinant host cell which expresses barstar from a promoter which is constitutively expressed in prokaryotic cells.

Claims: 41-43

A vector comprising an open reading frame 3' to a DNA fragment of no more than 30 base pairs, which DNA fragment comprise a ribosome binding site, **a SgfI recognition site**, and a sequence which, when present in mRNA enhances the binding of the mRNA to the small subunit of a eucaryotic ribosome; a vector comprising a SgfI recognition site, a sequence which comprises ATG and which sequence when present in mRNA, enhances the binding of the mRNA to the small subunit of a eucaryotic ribosome, and an open reading frame which begins at the ATG in the sequence;

Claims: 44-102, (116-119,121-126,128)-partially

A vector comprising a **SgfI recognition site 5' to a recognition site for a first restriction enzyme** which generates blunt ends; a vector comprising a first open reading frame which includes a SgfI recognition site and a recognition site which is not in the open reading frame for a restriction enzyme that has infrequent restriction sites in cDNAs or open reading frames from at least one species and generates blunt ends; a vector comprising a ribosome binding site which optionally overlaps by one nucleotide with a SgfI recognition site and a recognition site which is not in the open reading frame for a restriction enzyme that has infrequent restriction sites in cDNAs or open reading frames from at least one species and generates blunt ends; a vector comprising a first open reading frame which includes a recognition site for a first restriction enzyme that generates a 3' TA overhang and a recognition site for a second restriction enzyme that is not in the open reading frame generates blunt ends; a support comprising a plurality of recombinant vectors; a process to prepare said support; a library of recombinant cells comprising said recombinant vectors;

Claims: 103-114, (116-119,121-126)-partially

A vector comprising a first open frame which includes a **PmeI recognition site** and is linked at the 5' end by a recognition site for a first restriction enzyme that generates complementary single-strand DNA overhangs; a support comprising a plurality of recombinant vectors, wherein at least one recombinant vector was prepared by using said

vector including said PmeI site; a method to prepare said support; a library of recombinant cells comprising said recombinant vectors;

Claims: 115, 120, (121-126,128)-partially, 127, 129, 130
A support comprising a plurality of recombinant vectors, two or more of which comprise an open reading frame for a different polypeptide, wherein at least one recombinant vector comprises a promoter and an **open reading frame which is flanked by two exchange site**; a method to prepare said support; a library of recombinant cells comprising recombinant vectors or a library of recombinant vectors comprise an open reading frame for a different polypeptide; a method to prepare a plurality of mutagenized recombinant vectors: comprising providing DNAs comprising a plurality of mutagenized open reading frames flanked by two restriction enzyme sites for a first restriction enzyme which is a **hapaxoterministic restriction enzyme** and generates a first pair of non-self complementary single-stranded DNA overhangs; a library of recombinant cells or a library of recombinant vectors, a plurality of which recombinant vectors comprise said mutagenized recombinant vectors;

Claims: 131-141
A method for **performing genetic analysis**, comprising a) populating a database of genetic data with a plurality of genetic records; b) querying the database of genetic data to identify a first subset of genetic records; wherein each record has at least one recognition site for one predetermined restriction enzyme or for a restriction enzymes included in a set of predetermined restriction enzymes; and c) determining a set of statistics associated with the restriction enzyme recognition sites for at least a second subset of genetic records in the first subset; A computerized system for genetic analysis, comprising a database of genetic data; a processor; a set of one or more programs executed by the processor causing the processor to: query the database of genetic data to identify a first subset of genetic records; wherein each record has at least one recognition site for one predetermined restriction enzyme or for restriction enzymes included in a set of predetermined restriction enzymes, and; determine a set of statistics associated with the restriction enzyme recognition sites for at least a second subset of genetic records in the first subset;

They are not so linked as to form a single general inventive concept (Rule 13.1 PCT) for

the following reasons:

Methods of directional cloning of cDNAs by the use single restriction enzymes having a hapaxoterministic recognition site is well known in the prior art. J. Han and W. Rutter describe lambda-gt22S containing two unique in frame SfiI sites positioned in such way that a single cleavage site of the vector by SfiI generates two asymmetric cohesive ends. This vector is suitable for directional cloning of a full-length cDNA library which was generated by a primer adapter strategy for cDNA synthesis, The phage were screened on agar plates, and individual clones were isolated from the phage library, or "mini-prep" lambda DNA templates by the PCR, without subcloning steps (NAR 16:11837,1988). A. Zelenetz and R. Levy described a method of directionally cloning cDNA, by constructing a pair of vectors and devised a cDNA cloning strategy that improves upon previously published methods. The vectors, pLIB:AZ and pLIB:ZA, have two unique (distinct religation specificities; SfiI sites flanking a stuffer fragment which contains the tetracycline-resistance element. These vectors permit the directional cloning of cDNA in both sense (pLIB:AZ) and antisense (pLIB:ZA) orientations relative to the promoter for phage T3 RNA polymerase. cDNA that was synthesized using a primer with a 5' sequence of a SfiI.B site followed by an oligo(dT)16 3' tail was then ligated to an adaptor with the sequence of a SfiI (A) site produced directional molecules that could be cloned into the pLIB vectors (Gene 89, 123-127, 1990).

Furthermore, M Aebi et al. described the directional cloning of the RbetaG sequence inserted as PvuII-BglII fragment into the HincII- and BamHi-cleaved SP64 plasmid, and the cloning of 5'-truncated beta-globin genes by ligating TaqI-BglII fragments into the AccI- and BamHI-cleaved SP64 vectors (Cell 47, 555-565, 1986). Therefore, the directional subcloning of DNA fragments using two different vectors and four different restriction type II enzymes having infrequent restriction sites in said ORF, and generating ends, wherein the first and third restriction enzymes (PvuII and HiincII) and the second and fourth restriction enzymes (BglII and BamHI) can be ligated by combining the second vector with the first linear DNA fragment in a suitable buffer, is already described in the prior art and well known for a man skilled in the art.

Moreover, D. Bilcock et al. and US20030143522 described already vectors, comprising (I) one or more restriction enzyme recognition sites for SgfI, (ii) comprising an ATG sequence, and in addition to SgfI, a restriction enzyme recognition site for an enzyme producing blunt ended DNA. Said vector, once digested with SgfI and said restriction enzyme which

generates blunt ends generates a 3'-TA overhang at one site of the DNA fragment and a flush end on the other site (J. Bio. Chem 274, 36379-36386,1999).
WO9102077 describes an efficient cloning system that is particular useful for cloning cDNA copies of eukaryotic mRNAs and can direct the orientation of inserts in plasmid composite rectors with large cloning capacities. A genetic cloning vector comprising at least one replicon; and a site for inserting DNA segments to be cloned that includes at least two non-symmetrical restriction enzyme recognition sequences, wherein at least two of said non-symmetrical restriction enzyme recognition sequences are identical; and the first of said identical restriction enzyme recognition sequences is in the inverted orientation with respect to a second identical sequence, and said first and second identical restriction enzyme recognition sequences include greater than six positions having invariable DNA base pairs. Moreover, WO0107633 described a method for directional insertion of DNA, comprising: providing a recipient DNA vector having a restriction site which contains a degenerate recognition sequence and which generates cohesive ends when digested with its corresponding restriction endonuclease (RE), and digesting recipient DNA vector with RE corresponding to the restriction site, generating two cohesive ends on the digested vector.

In the view of the fact that methods for directional cloning are known; due to the fact that vectors for the directional cloning of multiple DNA sequences are already known, furthermore, due to the fact that vectors comprising a SgfI recognition site 5' to a recognition site for a first restriction enzyme which generates blunt ends are state of the art, and libraries of recombinant cells comprising recombinant vectors or a library of recombinant vectors are known, and moreover alternative methods of preparing a support comprising a plurality of recombinant vectors are well known, due to essential difference of the eight problems and at least nine different solutions and due to the fact that no other technical features can be distinguished which, in the light of the prior art, could be regarded as special technical features common to these solutions, the ISA is of the opinion that there is no single inventive concept underlying the plurality of claimed inventions of the present application in the sense of 13.1 PCT. Consequently there is lack of unity and different inventions, not belonging to a common inventive concept are formulated as the different subjects on the communication pursuant to Art. 17(3)(a), PCT.

Re Item V.

1 Reference is made to the following documents:

D1: AEBI M ET AL: "Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA." CELL. 21 NOV 1986, vol. 47, no. 4, 21 November 1986 (1986-11-21), pages 555-565, XP008052167 ISSN: 0092-8674

D2: MONACO L ET AL: "AN IN VITRO AMPLIFICATION APPROACH FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN MAMMALIAN CELLS" BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY, ACADEMIC PRESS, US, vol. 20, no. 2, October 1994 (1994-10), pages 157-171, XP001053058 ISSN: 0885-4513

D3: ZELENETZ A D ET AL: "DIRECTIONAL CLONING OF CDNA USING A SELECTABLE SFII CASSETTE" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 89, no. 1, 1990, pages 123-127, XP001019191 ISSN: 0378-1119

D4: HAN J H ET AL: "LAMBDAGT22S, A PHAGE EXPRESSION VECTOR FOR THE DIRECTIONAL CLONING OF CDNA BY THE USE OF A SINGLE RESTRICTION ENZYME SFII" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 16, no. 24, 1988, page 11837, XP001026354 ISSN: 0305-1048

D5: BILCOCK DENZIL T ET AL: "Reactions of type II restriction endonucleases with 8-base pair recognition sites" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 51, 17 December 1999 (1999-12-17), pages 36379-36386, XP002194222 ISSN: 0021-9258

D6: US-A-5 391 487 (KAPPELMAN ET AL) 21 February 1995 (1995-02-21)

D7: KAPPELMAN J R ET AL: "SgfI, a new type-II restriction endonuclease that recognizes the octanucleotide sequence 5'-GCGATCGC-3'" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 160, no. 1, 4 July 1995 (1995-07-04), pages 55-58, XP004042177 ISSN: 0378-1119

D8: US.2003/143522 A1 (PERLER FRANCINE B ET AL) 31 July 2003 (2003-07-31)

D9: WO 01/07633 A (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY OF AGRIC) 1 February 2001 (2001-02-01)

D10: WO 91/02077 A (THE UNITED STATES OF AMERICA, REPRESENTED BY THE S) 21 February 1991 (1991-02-21)

D11: BERGER S L: "Gene modification with hapaxoterministic restriction enzymes. Easing the way." METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.) 2001, vol. 160, 2001, pages 443-458, XP008052201 ISSN: 1064-3745

D12: BERGER S L ET AL: "PHOENIX MUTAGENESIS: ONE-STEP REASSEMBLY OF MULTIPLY CLEAVED PLASMIDS WITH MIXTURES OF MUTANT AND WILD-TYPE FRAGMENTS" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 214, 1993, pages 571-579, XP002043107 ISSN: 0003-2697

D13: BERGER S L: "Expanding the potential of restriction endonucleases: use of hapaxoterministic enzymes." ANALYTICAL BIOCHEMISTRY. OCT 1994, vol. 222, no. 1, October 1994 (1994-10), pages 1-8, XP002353097 ISSN: 0003-2697

D14: WO 91/05866 A (SCHERING CORPORATION) 2 May 1991 (1991-05-02)

D15: WILMS B ET AL: "High-cell-density fermentation for production of L-N-carbamoylase using an expression system based on the Escherichia coli rhaBAD promoter" BIOTECHNOLOGY AND BIOENGINEERING, WILEY & SONS, HOBOKEN, NJ, US, vol. 73, no. 2, 20 April 2001 (2001-04-20), pages 95-103, XP002228440 ISSN: 0006-3592

D16: STUMPP T ET AL: "EIN NEUES, L-RHAMNOSE-INDUZIERBARES EXPRESSIONSSYSTEM FUER ESCHERICHIA COLI" BIOSPEKTRUM, SPEKTRUM AKADEMISCHER VERLAG, DE, vol. 6, no. 1, 2000, pages 33-36, XP009004621 ISSN: 0947-0867

D17: EP-A-0 178 863 (SCHERING CORPORATION) 23 April 1986 (1986-04-23)

D18: EP-A-0 792 934 (COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH; DEPARTMENT OF BIOTECHNO) 3 September 1997 (1997-09-03)

D19: HARTLEY R W: "BARNASE AND BARSTAR EXPRESSION OF ITS CLONED INHIBITOR PERMITS EXPRESSION OF A CLONED RIBONUCLEASE" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB,

vol. 202, no. 4, 1988, pages 913-915, XP000993227 ISSN: 0022-2836

D20: YAZYNIN S A ET AL: "A PLASMID VECTOR WITH POSITIVE SELECTION AND DIRECTIONAL CLONING BASED ON AN CONDITIONALLY LETHAL GENE" GENE, ELSEVIER, AMSTERDAM, NL, vol. 169, no. 1, 1996, pages 131-132, XP002910844 ISSN: 0378-1119

D21: JUCOVIC MILAN ET AL: "In vivo system for the detection of low level activity barnase mutants" PROTEIN ENGINEERING, vol. 8, no. 5, 1995, pages 497-499, XP008055070 ISSN: 0269-2139

D22: CHEN M; NAGARAJAN V: "The roles of signal peptide and mature protein in Rnase (barnase) export from Bacillus subtilis." MOLECULAR & GENERAL GENETICS : MGG. GERMANY, JUN 1993, vol. 239, no. 3, June 1993 (1993-06), pages 409-415, XP002353285 ISSN: 0026-8925

D23: WO 01/55369 A (THE SCRIPPS RESEARCH INSTITUTE; THE NEUROSCIENCES INSTITUTE; MAURO, VI) 2 August 2001 (2001-08-02)

D24: MARTINEZ-SALAS E: "INTERNAL RIBOSOME ENTRY SITE BIOLOGY AND ITS USE IN EXPRESSION VECTORS" CURRENT OPINION IN BIOTECHNOLOGY, LONDON, GB, vol. 10, no. 5, 1999, pages 458-464, XP000943666 ISSN: 0958-1669

D25: DE 195 14 310 A1 (KLINIKUM DER ALBERT-LUDWIGS-UNIVERSITAET FREIBURG, 79106 FREIBURG, DE) 24 October 1996 (1996-10-24)

D26: CHAPPELL S A ET AL: "A 9nt segment of a cellular mRNA can function as an internal ribosome entry site (ires) and when present in linked multiple copies greatly enhances ires activity" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 97, no. 4, 15 February 2000 (2000-02-15), pages 1536-1541, XP002202271 ISSN: 0027-8424

D27: EP-A-1 184 462 (SMITHKLINE BEECHAM CORPORATION; SMITHKLINE BEECHAM PLC) 6 March 2002 (2002-03-06)

D28: DENNIS JONATHAN J ET AL: "Rapid generation of nested deletions by differential restriction digestion." BIOTECHNIQUES. AUG 2002, vol. 33, no. 2, August 2002 (2002-08), pages 310 , 312 , 314-315, XP002353098 ISSN: 0736-6205

D29: CSIRIK J ET AL: "A computer algorithm to determine the recognition site of

restriction enzymes." CABIOS. SEP 1987, vol. 3, no. 3, September 1987 (1987-09), pages 245-246, XP0008055387 ISSN: 0266-7061

D30: ELLROTT KYLE P ET AL: "Restriction enzyme recognition sequence search program." BIOTECHNIQUES. DEC 2002, vol. 33, no. 6, December 2002 (2002-12), pages 1322-1326, XP002353099 ISSN: 0736-6205

D31: VINCZE TAMAS ET AL: "NEBcutter: A program to cleave DNA with restriction enzymes." NUCLEIC ACIDS RESEARCH. 1. JUL 2003, vol. 31, no. 13, 1. July 2003 (2003-07-01), pages 3688-3691, XP002353100 ISSN: 1362-4962

2 INDEPENDENT CLAIM 1

2.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 1 is not new in the sense of Article 33(2) PCT.
Document **D1** discloses the directional cloning of the RbetaG sequence inserted as PvuII-BglII fragment into the HincII- and BamHi-cleaved SP64 plasmid, and the cloning of 5'-truncated beta-globin genes by ligating TaqI-BglII fragments into the AccI- and BamHI-cleaved SP64 vectors. Therefore, the directional subcloning of DNA fragments using two different vectors and four different restriction type II enzymes having infrequent restriction sites in said ORF, and generating ends, wherein the first and third restriction enzymes (PvuII and HiincII) and the second and fourth restriction enzymes (BglII and BamHI) can be ligated by combining the second vector with the first linear DNA fragment in a suitable buffer, is already described in the prior art.

3 INDEPENDENT CLAIM 14

3.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 14 is not new in the sense of Article 33(2) PCT.

D1 describes the cloning of BamHI-ECoRI fragments of the beta-globin genes containing the mutated region into the P327/SV40/MbetaG/RbetaG type A expression vector. The RbetaG gene was released by PvuII and BglII which can be

religated to a second vector comprising a second selectable marker gene which is different from the first selectable marker gene and non-essential DNA sequences, flanked by at least two restriction enzyme sites,

3.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 14 is not new in the sense of Article 33(2) PCT. Document **D3** described a method of directionally cloning cDNA, by constructing a pair of vectors and devised a cDNA cloning strategy that improves upon previously published methods. The vectors, pLIB:AZ and pLIB:ZA, have two unique (distinct religation specificities; SfiI sites flanking a stuffer fragment which contains the tetracycline-resistance element. These vectors permit the directional cloning of cDNA in both sense (pLIB:AZ) and antisense (pLIB:ZA) orientations relative to the promoter for phage T3 RNA polymerase. cDNA that was synthesized using a primer with a 5' sequence of a SfiI.B site followed by an oligo(dT)16 3' tail was then ligated to an adaptor with the sequence of a SfiI (A) site produced directional molecules that could be cloned into the pLIB vectors. Therefore, a vector system comprising: a first vector comprising a first selectable marker gene and a DNA sequence of interest, which DNA sequence of interest is flanked by at least two restriction enzyme sites, wherein at least one of the flanking restriction enzyme sites is a site for a first restriction enzyme which has infrequent restriction sites in cDNAs or open reading frames from at least one species and generates complementary single-strand DNA overhangs, wherein at least one of the flanking restriction enzyme sites is for a second restriction enzyme which has infrequent restriction sites in cDNAs or open reading frames from at least one species and generates ends that are not complementary to the overhangs generated by the first restriction enzyme wherein digestion of the first vector generates a first linear DNA fragment which lacks the first selectable marker gene but comprises the DNA sequence of interest, wherein the restriction enzyme sites are designed such that the first linear DNA fragment can be religated directly to a second vector
is already known in the prior art.

3.3 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 14 is not new in the sense of Article 33(2) PCT.
Document **D4** discloses lambda-gt22S containing two unique in frame SfiI sites

positioned in such way that a single cleavage site of the vector by SfiI generates two asymmetric cohesive ends. This vector is suitable for directional cloning of a full-length cDNA library which was generated by a primer adapter strategy for cDNA synthesis, The phage were screened on agar plates, and individual clones were isolated from the phage library, or "mini-prep" lambda DNA templates by the PCR, without subcloning steps. **D10** describes a genetic cloning system is which is particularly useful for cloning cDNA copies of eukaryotic mRNAS and can direct the orientation of inserts in plasmid composite vectors with large cloning capacities. Cleavage of such vector DNA, by the restriction enzyme SfiI creates two different non-symmetrical 3' extensions at the ends of vector DNA. Using a linker-primer and an adaptor, cDNA is prepared to have two different sticky ends which can be ligated to those of the vector. When the cDNA fragments and the vector DNAs are mixed, both the molecules can assemble without self-circularization due to base-pairing specificity.

4 DEPENDENT CLAIMS 2-5, 15-18, 26, 40

Dependent claims 2-5, 15-18, 26, 40 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).

5 INDEPENDENT CLAIM 27

5.1 The documents **D11** which is considered to represent the most relevant state of the art, discloses a class of restriction endonucleases called hapaxoterministic enzymes. A hapaxomer cleaves DNA outside the recognition site or within an interrupted "palindrome" at bases which are not specified producing fragments with asymmetric, staggered ends. Such termini are unique; in the general case, the protrusions of a fragment obtained with the aid of a

hapaxomer cannot self-hybridize, nor can the fragment be ligated to the vast majority of other fragments produced by the same enzyme. When the fragments generated by a hapaxoterministic enzyme are mixed, they can reassemble in only one

configuration--that of the original structure from which they were derived.

From this, the subject-matter of independent claim 28 differs in that **D11** does not describe the combining at least **two vectors** in a suitable buffer with one or more hapaxoterministic restriction enzymes to result in digestion.

5.1.1 The subject-matter of claim 27 is therefore novel (Article 33(2) PCT)
The problem to be solved by the present invention may be regarded as the provision of an alternative method of producing a vector suitable for expression of an amino acid sequence of interest.

5.1.2 The solution to this problem proposed in claim 27 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons: **D12** and **D13** describe already the ability to reunite once-contiguous fragments efficiently. This means that hapaxomers cleaving of DNA at many locations are virtually equivalent to restriction enzymes cutting at unique sites. These properties was exploited for applications such as site-specific mutagenesis which can be used with any gene in any vector. This method has been named "phoenix mutagenesis. Upon selection of the mutation site, the plasmid bearing the gene in question is cleaved with one or two restriction enzymes that generate fragments with random staggered ends. The enzymes are chosen to maximize the protrusions while minimizing the size of the fragment to be mutated. Ligation of the fragments reconstitutes the original plasmid. For a man skilled in the art, it is not obvious to combine the subject-matters of the prior art documents **D11** with **D12** respectively **D13,** to achieve the same result as in present application.

6 INDEPENDENT CLAIM 28

6.1 Document **D2**, which is considered to represent the most relevant state of the art, discloses the sequential directed cloning of the 2.3 kb HindIII-BamHI fragment from pSV2-neo into the HindIII- and BglII-cleaved pEMBL-Bgl plasmid,
From this, the subject-matter of independent claim 28 differs in that **D2** does not

describe the combining at least two vectors in a suitable buffer with one or more restriction enzymes to result in digestion.

6.1.1 The subject-matter of claim 28 is therefore novel (Article 33(2) PCT)
The problem to be solved by the present invention may be regarded as the provision of an alternative method of directional subcloning of DNA fragments.

6.1.2 The solution to this problem proposed in claim 28 of the present application is considered as involving an inventive step (Article 33(3) PCT) because for a man skilled in the art, it is not obvious to combine any of the subject-matters of the prior art documents to achieve the same result as in present application. The subject-matter of present claim comprises an improved and simplified method to directional clone a nucleic acid sequence of interest.

6.1.3 DEPENDENT CLAIMS 29-36

Claims 29-36 are dependent on claim 28 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

7 INDEPENDENT CLAIM 37

7.1 Document **D15** respectively **D16**, which is considered to represent the most relevant state of the art, discloses a high-cell-density fed-batch fermentation for the production of heterologous proteins in Escherichia coli was developed using the positively regulated Escherichia coli rhaBAD promoter. The expression system was improved by reducing of the amount of expensive L-rhamnose necessary for induction of the rhamnose promoter and by increasing the vector stability. Consumption of the inducer L-rhamnose was inhibited by inactivation of L-rhamnulose kinase encoding gene rhaB of Escherichia coli W3110, responsible for the first irreversible step in rhamnose catabolism.

From this, the subject-matter of independent claim 37 differs in that **D15** respectively

D16, does not describe the a rhamnose-inducible promoter operably linked to an open reading frame for a heterologous RNA polymerase.

7.1.1 The subject-matter of claim 37 is therefore novel (Article 33(2) PCT)
The problem to be solved by the present invention may be regarded as the provision of an alternative method of inducing expression of DNA sequence of interest in a host cell.

7.1.2 The solution to this problem proposed in claim 37 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons: **D14**, **D17** and/or **D18** describe a DNA sequence comprising a bacteriophage T7 promoter linked in correct reading frame to a gene encoding a heterologous protein or polypeptide; a DNA sequence comprising an inducible promoter linked in correct reading frame to a gene encoding a mutant bacteriophage T7 RNA polymerase which is capable of binding to a T7 promoter and carrying out transcription without being lethal to a bacterial host expressing the gene. A man skilled in the art incentive to improve the method of **D15**, would combine the technical feature of **D15** (respectively **D16**) with any of the subject-matters of the prior art documents **D14**, **D17** and/or **D18** to achieve the same result as in present application.

8 DEPENDENT CLAIM 38

Dependent claim 38 does not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).

9 INDEPENDENT CLAIM 39

9.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 39 is not new in the sense of Article 33(2) PCT.
Documents **D19** and/or **D20** describe the expression of barnase and barstar.

When the wild-type gene for barnase (with the phoA promoter-signal sequence) is reconstructed from its previously cloned parts on the same plasmid as the barstar gene, the lethal effect of its expression is suppressed. A plasmid has been devised which directs the secretion of 100 mg per active barnase liter by E. coli and another which provides large (500 to 1000 mg/l) yields of barstar.

9.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 39 is not new in the sense of Article 33(2) PCT.

Document **D21** discloses the design of a new tightly controlled barnase system which allows the existence of the barnase gene in host cells without a signal sequence. When expression of barnase is turned on by gene inversion in vivo, the lethal effect of barnase (or its mutants) is not compromised by coexpression of its polypeptide inhibitor (barstar).

9.3 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 39 is not new in the sense of Article 33(2) PCT.

Document **D22** describes the roles of signal peptide and mature protein in RNase (barnase) export from Bacillus subtilis. The rate of secretion of barnase from B. subtilis was improved by replacement of the barnase signal peptide with a heterologous signal peptide. However, the barnase signal peptide exported E. coli alkaline phosphatase faster than mature barnase.

10 INDEPENDENT CLAIMS 41 AND 43

10.1 **D23** describes a synthetic translational regulatory element useful for altering translational activity which comprising oligonucleotides with translational regulatory activity and internal ribosome entry site activity. An expressible polynucleotide comprises a first cistron encoding a polypeptide that enhances protein stability or cell viability, and a second cistron encoding a polypeptide of interest. The second cistron is operatively linked to the first cistron, and the expressible polynucleotide comprises an IRES element, which is operatively linked to the first cistron or the second cistron or both. **D24** describes an internal ribosome entry site and its use in expression vectors. Additionally, progress is

made in the understanding the parameters that influence start codon selection by the rational design of bicistronic expression vectors. **D25** discloses vectors for transfection of cells comprising multiple cistron containing regulatory elements, a gene to be expressed, an internal ribosome entry sequence and a selectable marker gene. The IRES ensures that the gene to be expressed and the selectable marker gene are transcribed under the control of a single promoter into a single mRNA molecule. Examples indicate that this improves transfection efficiency when the vector has a 3'-NTS. **D26** describes a 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity.

Any one of the documents **D23-D26**, which is considered to represent the most relevant state of the art, discloses a vector comprising an open reading frame 3' to a DNA fragment of no more than 30 base pairs, which DNA fragment comprises a ribosome binding site and a sequence which, when present in mRNA, enhances the binding of the mRNA to the small subunit of a eucaryotic ribosome.

From this, the subject-matter of independent claims 41 and 43 differs in that **D23** to **D26**, do not describe the a SgfI recognition site.

10.1.1 The subject-matter of claims 41 and 43 is therefore novel (Article 33(2) PCT) The problem to be solved by the present invention may be regarded as the provision of an alternative vector comprising a SgfI recognition site linked to an IRES and an additional ribosome binding site.

10.1.2 The solution to this problem proposed in claim 37 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons: **D5, D6** and **D7** describe already the restriction endonuclease SgfI isolated from Streptomyces griseoruber, used for specifically cleaving an octa:nucleotide recognition sequence. Due to its 8 bp length, the recognition sequence is found infrequently along strands of DNA as compared to recognition sequences of fewer base pairs so that fewer and larger fragments of DNA can be generated. SgfI is complementary to PvuI and they share the same cleavage site,

In the absence of a surprising technical effect associated with said alternative vector construct, the choice of a SgfI recognition site is considered to be arbitrary.

11 DEPENDENT CLAIM 42

Dependent claim 42 does not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step (Article 33(3) PCT).

12 INDEPENDENT CLAIMS 44, 56, 59, 71, 78 AND 79

12.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 44, 56, 59, 71, 78 AND 79 is not new in the sense of Article 33(2) PCT. Document **D5** describes reactions of type II restriction endonucleases with 8-base pair recognition sites, which were tested on plasmids that have either one or two copies of the relevant sequence. **SgfI** (T/A overhang), **SrfI** (blunt end), FseI, PacI, **PmeI** (blunt end), Sse8781I, and SdaI all acted through equal and independent reactions at each site. Furthermore, D5 describes the vector pDB8 comprising a SgfI recognition site 5' to a recognition site for a first restriction enzyme (SrfI) which generates blunt ends.

13 INDEPENDENT CLAIMS 73, 86 AND 87

13.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 73, 86 AND 87 is not new in the sense of Article 33(2) PCT. **D4** describes Lambda gt22S, a phage expression vector for the directional cloning of cDNA by the use of a single restriction enzyme SfiI. Moreover, to increase the efficiency of directionally cloning cDNA, **D3** constructed a pair of vectors and devised a cDNA cloning strategy that improves upon previously published methods. The vectors, pLIB: AZ and pLIB: ZA, have two unique (distinct religation specificities;

GGCCN/NNNNGGCC) SfiI sites (SfiI.A and SfiI.B)

flanking a stuffer fragment which contains the tetracycline-resistance element. These vectors permit the directional cloning of cDNA in both sense (pLIB: AZ) and antisense (pLIB: ZA) orientations relative to the promoter for phage T3 RNA polymerase. cDNA that was synthesized using a primer with a 5' sequence of a SfiI.B site followed by an oligo(dT)16 3' tail was then ligated to an adaptor with the sequence of a SfiI. **D10** describes genetic cloning system is disclosed which is

particularly useful for cloning cDNA copies of eukaryotic mRNAs and can direct the orientation of inserts in plasmid composite vectors with large cloning capacities. Cleavage of such vector DNA, by the restriction enzyme SfiI creates two different non-symmetrical 3' extensions at the ends of vector DNA.

14 DEPENDENT CLAIMS 45, 46, 49, 50, 51, 60, 61, 64, 65, 80, 81 AND 116-119

Dependent claims 45, 46, 49, 50, 51, 60, 61, 64, 65, 80, 81 and 116-119 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).

15 INDEPENDENT CLAIMS 57 AND 72

The documents **D5**, which is considered to represent the most relevant state of the art, discloses a vector comprising an open reading frame flanked by a first restriction endonuclease SgfI that generates a 3'TA overhang which is 5' to a recognition site for a second restriction enzyme SrfI which generates a blunt end.

From this, the subject-matter of independent claims 57 and 72 differs in that **D5** does not describe a ribosome binding site which overlaps by one nucleotide with the SgfI recognition site.

15.1.1 The subject-matter of claim 57 and 72 is therefore novel (Article 33(2) PCT)
The problem to be solved by the present invention may be regarded as the

provision of an alternative expression vector. The subject-matter of present claims 57 and 72 comprises an improved expression vector sequence suitable for a simplified method to directional clone a nucleic acid sequence of interest.

16 INDEPENDENT CLAIMS 82, 83 AND 84

The present application meets the criteria of Article 33(1) PCT, because the subject-matter of the claims 82, 83, 84 and appear to be new in the sense of Article 33(2) PCT.

17 DEPENDENT CLAIMS 85 and 121-126

Claims 85 and 89-100 are dependent on claim 84 and 88 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

18 INDEPENDENT CLAIM 88

18.1 The document **D3** which is considered to represent the most relevant state of the art, discloses a method to introduce two different recognition sites for an restriction enzyme to the ends of an open reading frame.

From this, the subject-matter of independent claim 88 differs in that **D3** does not describe the a SgfI and blunt end recognition sites.

18.1.1 The subject-matter of claim 88 is therefore novel (Article 33(2) PCT). The problem to be solved by the present invention may be regarded as the provision of a method to introduce two alternative recognition sites for different restriction enzymes to the end of an open reading frame.

18.1.2 The solution to this problem proposed in claim 88 of the present application

cannot be considered as involving an inventive step (Article 33(3) PCT), for the following reasons: **D4, D9** and **D10** describe already alternative methods to introduce two alternative recognition sites for different restriction enzymes to the end of an open reading frame, and in the absence of a surprising technical effect associated with said alternative method, the choice of a SgfI recognition site and blunt end restriction site for different restriction enzymes is considered to be arbitrary.

19 DEPENDENT CLAIMS 89-97 AND 99-100

Dependent claims 89-97 and 99-100 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step (Article 33(3) PCT).

20 INDEPENDENT CLAIMS 103, 107, 110, 112, 113 AND 114

20.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 103 is not new in the sense of Article 33(2) PCT. Document **D5** describes reactions of type II restriction endonucleases with 8-base pair recognition sites, which were tested on plasmids that have either one or two copies of the relevant sequence. **SgfI** (T/a overhang), **SrfI** (blunt end), FseI, PacI, **PmeI** (blunt end), Sse8781I, and SdaI all acted through equal and independent reactions at each site. Furthermore, **D5** describes the vector pAB1 comprising a first open reading frame which includes a **PmeI** recognition site and is flanked at the 5' end by a recognition site for a first restriction enzyme that generates complementary single stranded DNA overhangs (**AscI, PacI** and/or **Sse8387I**).

20.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 103, 107, 110, 112, 113 and 114 is not new in the sense of Article 33(2) PCT. Document **D27** describes a method for cloning substantially all of the genes of a prokaryotic organism's genome into expression vectors comprising

an inducible promoter to form an open reading frame expression library, inducing expression of a copy of the library of genes with an inducer, contacting the copy of the library of induced genes and a copy of the library of uninduced genes with antimicrobial compound which kills the cells with non-target genes; and determining the target gene of the antimicrobial compound by identifying the gene conferring cell survival of the treatment of AB. Moreover, **D27** discloses an inducible expression vector pYH4 comprising the cloning sites for PmeI and AscI sites.

20.3 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 103 and 110 is not new in the sense of Article 33(2) PCT. **D28** describes a method for generating nested deletions in DNA that exploits the difference in frequency of restriction sites recognized by compatible restriction endonucleases. a cloning vector was constructed that contains no common blunt-end or RsaI restriction sites and two 8-bp blunt-end restriction sites flanking a commodious multiple cloning site. DNA fragments are cloned into the multiple cloning site using blue-white selection, and nested deletions are generated by digesting the resulting plasmid with either SwaI or PmeI and partially digesting the insert DNA with RsaI.

21 DEPENDENT CLAIMS 105, 106, 108, 109, 111 AND 116-119, 121-126

Dependent claims 105, 106, 108, 109, 111, 116-119 and 121-126 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT).

22 INDEPENDENT CLAIMS 115, 120, 127, 129 AND 130

22.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 115, 120, 129 and 130 is not new in the sense of Article 33(2) PCT. Document **D9** describes a method for directional insertion of DNA, comprising providing a recipient DNA vector having a restriction site which contains a

degenerate recognition sequence and which generates cohesive ends when digested with its corresponding restriction endonuclease (RE), and digesting recipient DNA vector with RE corresponding to the restriction site, generating two cohesive ends on the digested vector

22.2 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 115, 120, 129 and 130 is not new in the sense of Article 33(2) PCT. Document **D4** describes Lambda gt22S, a phage expression vector for the directional cloning of cDNA by the use of a single restriction enzyme SfiI. Moreover, to increase the efficiency of directionally cloning cDNA, **D3** constructed a pair of vectors and devised a cDNA cloning strategy that improves upon previously published methods. The vectors, pLIB: AZ and pLIB: ZA, have two unique (distinct religation specificities; GGCCN/NNNNGGCC) SfiI sites (SfiI.A and SfiI.B) flanking a stuffer fragment which contains the tetracycline-resistance element. These vectors permit the directional cloning of cDNA in both sense (pLIB: AZ) and antisense (pLIB: ZA) orientations relative to the promoter for phage T3 RNA polymerase. cDNA that was synthesized using a primer with a 5' sequence of a SfiI.B site followed by an oligo(dT)16 3' tail was then ligated to an adaptor with the sequence of a SfiI. **D10** discloses a genetic cloning system is disclosed which is particularly useful for cloning cDNA copies of eukaryotic mRNAS and can direct the orientation of inserts in plasmid composite vectors
with large cloning capacities. Cleavage of such vector DNA, by the restriction enzyme SfiI creates two different non-symmetrical 3' extensions at the ends of vector DNA.

22.3 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 115, 120, 127, 129 and 130 is not new in the sense of Article 33(2) PCT. Documents **D11-D13** describe a method for site-specific mutagenesis which can be used with any gene in any vector has been devised. This method has been named "phoenix mutagenesis." The enzymes are chosen to maximize the protrusions while minimizing the size of the fragment to be mutated. Ligation of the fragments reconstitutes the original plasmid. Mutations are obtained by allowing mutant fragments, added in 10-fold molar excess, to
compete with their wild-type counterparts for a place in the reassembled vector.

23 DEPENDENT CLAIMS 121-126 AND 128

Dependent claim 121-126 and 128 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step (Article 33(3) PCT).

24 INDEPENDENT CLAIM 131

The present application meets the criteria of Article 33(1) PCT, because the subject-matter of the claim 131 appear to be new in the sense of Article 33(2) PCT.

25 DEPENDENT CLAIMS 132-140

Claims 132-140 are dependent on claim 131 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

26 INDEPENDENT CLAIM 141

26.1 The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 141 is not new in the sense of Article 33(2) PCT.

Document **D29** describes an algorithm to determine the type-II restriction endonucleases' recognition site knowing the digested DNA sequence and fragment lengths in an actual case. **D30** discloses restriction enzyme recognition sequence search program. To simplify this process, they developed a plasmid transformation method along with a computer program named RM search that determines the exact recognition sequences for given restriction and modification systems. **D31** describes a NEBcutter program available via a web server (http://tools.neb.com/NEBcutter) that will accept an input DNA sequence and produce a comprehensive report of the restriction enzymes that will cleave the sequence. It produces a variety of outputs including restriction enzyme maps,

theoretical digests and links into the restriction enzyme database, REBASE (http://www.neb.com/rebase).

Re Item VIII.

CLARITY (ART 6 PCT)

1 Concerning the objections made for clarity, Art. 6 PCT requires amongst other things that the claims, which define the matter for which protection is sought be clear. This has to be interpreted as meaning not only that a claim from the technical point of view must be comprehensible, but that it must define clearly the object of the invention, that is to say indicate all the essential technical features, which are necessary to obtain the desired effect or, differently expressed, which are necessary to solve the technical problem with which the application is concerned without undue experimentation.

1.1 The supports described in claims 73-77, 79-81, 110-111 and 115-119 are not rendered novel merely by the fact that they are produced or isolated by means of a new process (cf Guidelines C III 4.7 (b)). Furthermore, the claims 73, 79, 110 and 115 are not clear to Art. 6 PCT. In the claims the applicant attempts to define the subject-matter (**"a support comprising a plurality of recombinant cells.."**) in terms of the result to be achieved (process of manufacture) (**"..are formed by ligation of ..."**) .

1.2 The recombinant vector described in claim 78 is not rendered novel merely by the fact that they are produced or isolated by means of a new process (cf Guidelines C III 4.7 (b)). Furthermore, said claim is not clear under Art. 6 PCT. In the claims the applicant attempts to define the subject-matter (**"a recombinant vector.."**) in terms of a the result to be achieved (**"..prepared by ..."**) .

1.3 The libraries of recombinant cells comprising recombinant vectors described in claims 86, 87, 102, 113, 114, 129 and 130 are not rendered novel merely by the fact that they are produced or isolated by means of a new process (cf Guidelines C III 4.7 (b)). Furthermore,

the claims 86, 87, 102, 113, 114, 129 and 130 are not clear to Art. 6 PCT. In the claims the applicant attempts to define the subject-matter (**"a library of recombinant cells.."**) in terms of a the result to be achieved (**"..are formed by ligation of ..."**) .

1.4 The recombinant cells described in claims 101 are not rendered novel merely by the fact that they are produced or isolated by means of a new process (cf Guidelines C III 4.7 (b)). Furthermore, said claim is not clear under Art. 6 PCT. The claims attempts to define the subject-matter (**"Recombinant cells.."**) in terms of a process of manufacture (**"..are formed by ..."**) .

1.5 The present application lacks clarity as required by Art. 6 PCT in that the matter for which protection is sought is not clearly defined. The applicant discloses a set of claims, in which the subject-matter of the independent claims 44, 56, 57, 59, 71, 72, 103 and 107 is presented as a "DESIDERATUM" par excellence. The expression: **" a vector ... once digested with ...and ligated to..."** the applicant is a and attempts to define the subject-matter in terms of a the result to be achieved. In this instance, however, such a formulation in the present case is not allowable because it appears possible to define the subject-matter in more concrete terms, viz. in terms how the product is to be achieved.

2 The present application lacks clarity as required by Art. 6 PCT. The claims as a whole are not clear and concise and thus do not fulfil the requirements of Art. 6 PCT. In the present case, the expression **"..optionally.."** claim 1, 14, 27 and 28 has been considered as having no limiting effect on the scope of said claim (see EPC Guidelines C-III 4.6).